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The Death of Ouabain-Treated Renal Epithelial C11-MDCK Cells is Not Mediated by Swelling-Induced Plasma Membrane Rupture

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Abstract This study examined the role of cell volume modulation in plasma membrane rupture and death documented in ouabain-treated renal epithelial cells. Long-term exposure to ouabain caused massive death of C11-MDCK (Madin-Darby canine kidney) epithelial cells, documented by their detachment, chromatin cleavage and complete loss of lactate dehydrogenase (LDH), but did not affect the survival of vascular smooth muscle cells (VSMCs) from the rat aorta. Unlike the distinct impact on cell survival, 2-h exposure to ouabain led to sharp elevation of the $[Na^+]_i/[K^+]_i$ ratio in both cell types. A similar increment of Na_i^+ content was evoked by sustained inhibition of Na^+,K^+-ATPase in K^+-free medium. However, in contrast to ouabain, C11-MDCK cells survived perfectly during 24-h exposure to K^+-free medium. At 3 h, the volume of ouabain-treated C11-MDCK cells and VSMCs, measured by the recently developed dual-image surface reconstruction technique, was increased by 16 and 12%, respectively, whereas 5–10 min before the detachment of ouabain-treated C11-MDCK cells, their volume was augmented by ~30–40%. To examine the role of modest swelling in the plasma membrane rupture of ouabain-treated cells, we compared actions of hypotonic medium on volume and LDH release. We observed that LDH release from hyposmotically swollen C11-MDCK cells was triggered when their volume was increased by approximately fivefold. Thus, our results showed that the rupture of plasma membranes in ouabain-treated C11-MDCK cells was not directly caused by cell volume modulation evoked by Na^+,K^+-ATPase inhibition and inversion of the $[Na^+]_i/[K^+]_i$ ratio.

Keywords Na^+,K^+-ATPase · Ouabain · Renal epithelium · Vascular smooth muscle · Cell volume · Plasma membrane integrity · Cell death

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Introduction

Na^+,K^+-ATPase, a heterodimer consisting of catalytic α- and regulatory β-subunits, plays a key role in the maintenance of electrochemical gradients of monovalent cations across the plasma membrane in all types of nucleated animal cells studied so far. Numerous investigations have demonstrated that ouabain and other cardiotonic steroids (CTSs) abolish transmembrane Na^+ and K^+ gradients via the inhibition of catalytic $\alpha1$–$\alpha4$ isoforms which, in turn, leads to the modulation of diverse Na_i^+,K_i^+-dependent cell functions, such as electrical membrane potential, cell volume, transepithelial movement of salt and osmotically obliged water, Na^+ symport with glucose, amino acids and nucleotides (Scheiner-Bobis 2002). In contrast to the universal impact on the above-listed cell functions, long-term treatment with CTS affects cell survival in a tissue-specific manner. Thus, CTS had no effect on the survival of rhesus monkey renal epithelial cells (Contreras et al. 1995, 1999), human lymphocytes (Falciola et al. 1994), rat vascular smooth muscle cells (VSMCs) (Orlov et al. 1999, 2001), rat astrocytes (Akimova et al. 2006a) and Jurkat cells (Panayiotidis et al.

2010) but resulted in massive death of epithelial cells from the Madin-Darby canine kidney (MDCK) (Ledbetter et al. 1986; Bolivar et al. 1987) and endothelial cells from the porcine aorta (Orlov et al. 2004).

Two major, morphologically distinct modes of cell death have been documented in tissue slices. The death of clustering, neighboring cells under complement attack, severe hypoxia, hyperthermia, lytic viral infection or exposure to toxins or poisons has been known as *necrosis,* a term introduced more than 100 years ago by Virchow. Necrotic stimuli evoked cell swelling accompanied by chromatin flocculation, endoplasmic reticulum dilatation and mitochondrial swelling with the inner membrane shrunken away from the outer membrane. This process is terminated by plasma membrane disruption and lysosomal enzyme release, which elicit inflammatory reactions and digestion of damaged cells by macrophages. In contrast to necrosis, cells undergoing apoptosis exhibit modest shrinkage rather than swelling that is terminated by nucleus fragmentation and the formation of so-called apoptotic bodies. At this point, apoptotic cells are recognized and digested by recruited macrophages and neighboring cells (Wyllie 1981). Such contrasting volume behavior was classified by Okada et al. (2001) as necrotic volume increase and apoptotic volume decrease.

Several biochemical markers, e.g., cytochrome *c* release from mitochondria, activation of cysteine proteases with aspartate substrate specificity (caspases), phosphatidylserine exposure on the plasma membrane outer surface, chromatin cleavage and DNA degradation with the appearance of multiple 180–200 bp fragments (so-called DNA laddering), have been defined in cells undergoing "classical" apoptosis (Duvall and Wyllie 1986). It should be underlined, however, that cytochrome *c* release is also observed in Jurkat cells enduring morphologically defined necrosis in the presence of oxidative phosphorylation inhibitors (Heiden et al. 1997). Moreover, caspase-3 activation occurs in viable lymphocytes as an obligatory step of T-cell receptor activation (Alam et al. 1990), and its suppression delays the development of necrosis caused by chemically induced hypoxia or toxins (Shimizu et al. 1996; Warny and Kelly 1999). In several cell types, morphologically defined apoptosis occurs in the absence of internucleosomal DNA cleavage, whereas both chromatin cleavage and DNA laddering are seen in cells undergoing necrosis (Columbano 1995; Dong et al. 1997). Phosphatidylserine exposure results from elevation of $[Ca^{2+}]_i$ (Zhou et al. 1997), i.e., a process that occurs in diverse physiological and pathophysiological conditions, including necrosis. The insufficiency of biochemical criteria for precise classification of the modes of cell death is considered in detail elsewhere (Kroemer et al. 2005).

The demise of CTS-treated epithelial and endothelial cells bears combined markers of canonical necrosis (modest cell swelling, negligible labeling with nucleotides in the presence of terminal transferase, nuclei staining with cell-impermeable dyes, such as propidium iodide) and apoptosis (nuclear condensation in cells stained with cell-permeable dyes such as Hoechst 33342, chromatin cleavage, caspase-3 activation). However, in contrast to classical apoptosis, the death of ouabain-treated MDCK cells is insensitive to the pan-caspase inhibitor z-VAD.fmk (Contreras et al. 1999; Pchejetski et al. 2003; Orlov et al. 2004). The dearth of definitive apoptosis vs. necrosis biochemical markers in CTS-treated cells is likely due to the fact that apoptosis and necrosis are not alternative "either/or" mechanisms but appear to be the opposite ends of a spectrum of cell death pathways (Kroemer et al. 2005).

In the absence of specific and selective markers, an opposite cell volume behavior seems to be a sole universal criterion to distinguish apoptosis vs. necrosis. Moreover, in accordance with the chemo-osmotic model (Armstrong 2003), cell swelling resulting from dissipation of Gibbs-Donnan equilibrium might be sufficient to trigger plasma membrane rupture (Carini et al. 1999; Okada et al. 2001). To examine this hypothesis, we undertook the recently developed dual-image surface reconstruction (DISUR) technique (Boudreault and Grygorczyk 2004) to compare the kinetics of cell volume modulation by ouabain and hypotonic shock in CTS-sensitive C11-MDCK cells resembling intercalated cells from collecting ducts and CTS-resistant VSMCs. Data obtained in our study argue against an impact of cell swelling on plasma membrane rupture seen in CTS-sensitive cells.

Methods

Cell Culture

C11-MDCK cells resembling intercalated cells from the collecting ducts of the Madin-Darby canine kidney (Gekle et al. 1994; Bourcier et al. 2002) and a cell line derived from VSMCs of the rat aorta and possessing the highest expression of smooth muscle-specific α-actin, SM22 protein and myosin light chain kinase as well as the highest sensitivity to angiotensin II and endothelin-1 (Davis et al. 2003) were used in this study. Cells were maintained in culture as described in detail elsewhere (Davis et al. 2003; Pchejetski et al. 2003), and their morphology (Davis et al. 2003) was evaluated by phase-contrast microscopy at ×100 magnification without preliminary fixation.

Cell Volume

Cell volume was measured in substrate-attached cells with an improved version of the DISUR technique described previously in detail (Fels et al. 2009). The method involves

3D reconstruction of the cell shape based on two conventional microscopic cell images acquired in two perpendicular directions. Side-view and top-view cell images were acquired by two independent, miniature, charge-coupled cameras (Moticam 350; Motic Instruments, Richmond, Canada) with Motic software at 10- to 60-s intervals to closely follow rapid volume changes. The images served to generate a set of topographical curves of the cell surface from its digitized side-view profile and base outline. Cell volume was calculated from the reconstructed cell topographical model with MATLAB (Math Works, Natick, MA). Although DISUR provides absolute cell volume values, data plots in the present study show relative volume normalized to initial intact cell volume in physiological solution. Cells seeded on coverslips were mounted in a custom-made flow-through imaging chamber and perfused at a rate of 1–2 ml/min with HEPES-buffered isotonic medium A at 37°C. Medium A was composed (in mM) of 135 NaCl, 5 KCl, 1.2 $MgSO_4$, 1.3 $CaCl_2$, 1.2 Na_2HPO_4, 10 D-glucose and 10 HEPES (pH 7.4, adjusted with NaOH). In K^+-free medium, KCl was substituted with an equimolar amount of NaCl. In some experiments, medium osmolarity was reduced by ~98% by omitting NaCl, KCl, Na_2HPO_4 and glucose.

Intracellular Exchangeable Na^+ and K^+ Content

Intracellular exchangeable Na^+ and K^+ content was measured as the steady-state distribution of extra- and intracellular ^{22}Na and ^{86}Rb (Orlov et al. 1999). Briefly, cells were incubated for 5 h in media with different Na, K and Cl concentrations, with or without ouabain, and in the presence of 0.5 μCi/ml $^{86}RbCl$ or 2 μCi/ml $^{22}NaCl$. At the end of incubation, they were transferred onto ice, washed four times with 2 ml of ice-cold medium W and lysed with SDS/EDTA mixture. Radioactivity of the incubation medium and cell lysate was measured, and intracellular cation content was calculated as A/am, where A was the radioactivity of the sample (cpm), a was the specific radioactivity of Na^+ and K^+ (^{86}Rb) in the medium (cpm/nmol) and m was protein content (mg).

Cell Attachment, Chromatin Cleavage and Lactate Dehydrogenase Release

Cell attachment, chromatin cleavage and lactate dehydrogenase (LDH) release were analyzed to estimate the death of ouabain-treated cells. To quantify cell detachment, cells were grown in 24-well plates in the absence or presence of ouabain, and the protein content of cells attached to plastic supports after three washes with 2-ml aliquots of medium W containing 100 mM $MgCl_2$ and 10 mM HEPES-Tris buffer (pH 7.4) was measured by the modified Lowry method. A chromatin cleavage assay was performed as described previously (Orlov et al. 1999). Shortly thereafter, cells grown in 24-well plates were supplied with DMEM containing serum and 0.2–0.5 μCi/ml [^{3}H]-thymidine. After 24 h, they were washed twice with 2 ml of DMEM and incubated for 24 h in isotope-free, serum-containing DMEM. They were then washed with PBS and incubated in 0.5 ml of isotope-free medium of different compositions for the next 18–24 h. To analyze chromatin fragmentation, the plates were transferred onto ice, and 1 ml of ice-cold lysed buffer (10 mM EDTA, 10 mM Tris-HCl, 0.5% Triton X-100, pH 8.0) was added. After 15 min, the cell lysate was collected, centrifuged (12,000 rpm, 10 min) and transferred for the measurement of radioactivity in a liquid scintillation spectrometer (fraction F_1). Radioactivity remaining in sediments and wells was extracted with 1% SDS/4 mM EDTA mixture (fraction F_2). The relative content of intracellular chromatin fragments was quantified as the percentage of total [^{3}H]-labeled DNA: $F_1/(F_1 + F_2)^{-1} \times 100\%$. To assess LDH activity, 0.5 ml of incubation medium was mixed with the same volume of 1% Triton X-100 and attached cells were solubilized with 0.5% Triton X-100. Then, 300 μl of samples were transferred to 2 ml of buffer containing 50 mM Tris-HCl (pH 7.4), 50 mM KCl, 0.075 mM NADH (β-nicotinamide-adenine dinucleotide, reduced) and 1 mM sodium pyruvate. The kinetics of NADH degradation were monitored by a spectrofluorimeter at $\lambda_{ex} = 349$ nm and $\lambda_{em} = 420$ nm (slits 4 and 20 nm, respectively). LDH release was calculated as a percentage of enzyme activity in the incubation medium.

Chemicals

Methyl-[^{3}H]-thymidine, $^{22}NaCl$ and ^{86}Rb were purchased from Isotope (St. Petersburg, Russia). DEVD-AMC, DEVD-CHO and z-VAD.fmk were obtained from Biomol Research Laboratories (Plymouth Meeting, PA). The remaining chemicals were procured from GIBCO BRL (Gaithersburg, MO), Calbiochem (La Jolla, CA), Sigma (St. Louis, MO) and Anachemia (Montreal, Canada).

Results

Actions of Ouabain and K^+-Free Medium on Intracellular Content of Monovalent Cations and Cell Survival

Previously, we reported that the addition of 3 μM ouabain completely blocked Na^+,K^+-ATPase in C11-MDCK cells (Akimova et al. 2005a). Table 1 shows that 5-h incubation with 3 μM ouabain resulted in ~13-fold elevation of Na_i^+ content and attenuation of K_i^+ content by approximately ninefold. It is well documented that the affinity of the

Table 1 Effect of ouabain and K^+-free medium on Na_i^+ and K_i^+ content in renal epithelial cells (C11-MDCK) and VSMCs

Medium	C11-MDCK		VSMC	
	Na_i^+ (nmol mg protein)	K_i^+ (nmol mg protein)	Na_i^+ (nmol mg protein)	K_i^+ (nmol mg protein)
Control (medium A)	72 ± 13	834 ± 89	64 ± 21	896 ± 97
Medium A + ouabain[a]	933 ± 103*	91 ± 21*	895 ± 120*	80 ± 19*
K^+-free medium	789 ± 89	ND	786 ± 99	ND
K^+-free medium + ouabain[a]	1,004 ± 156	ND	962 ± 78	ND

Na_i^+ and K_i^+ contents were measured after 5-h incubation of cells in control or K^+-free medium

ND values not determined

* $P < 0.001$ compared to controls

[a] In experiments with C11-MDCK cells and VSMCs, ouabain was added at concentrations of 3 and 3,000 μM, respectively. Means ± SE are reported from experiments performed in quadruplicate

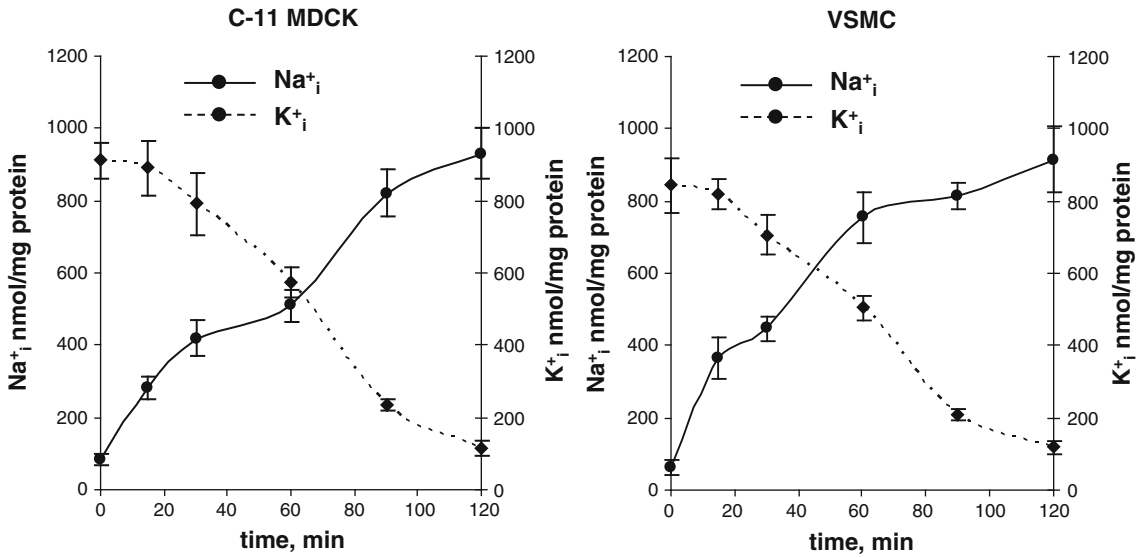

Fig. 1 Kinetics of modulation of intracellular Na^+ and K^+ content by ouabain in renal epithelial (C11-MDCK) cells and VSMCs. In C11-MDCK cells and VSMCs, ouabain was added at concentrations of 3 and 3,000 μM, respectively. Means ± SE from experiments performed in quadruplicate are shown

ubiquitous α1-Na^+,K^+-ATPase isoform for CTS in rodents is ~1,000-fold less than in other mammalian species (Lingrel et al. 2007). Considering this, in experiments with rat VSMCs, we increased ouabain concentration to 3,000 μM. At this ouabain concentration, 5-h incubation of VSMCs led to ~14-fold gain of Na_i^+ and 11-fold loss of K_i^+ compared to control values (Table 1). Comparison of Table 1 and Fig. 1 indicates complete inversion of the $[Na^+]_i/[K^+]_i$ ratio in both cell types within 2 h of ouabain addition. As predicted, ~10-fold $[Na^+]_i$ elevation was also detected after 5-h incubation of C11-MDCK cells and VSMCs in K^+-free medium. Addition of ouabain increased $[Na^+]_i$ content by <30% in this medium (Table 1).

Consistent with previous investigations (Pchejetski et al. 2003), we observed that 24-h inhibition of Na^+,K^+-ATPase by ouabain resulted in the accumulation of rounded, floating C11-MDCK cells (Fig. 2) as well as in marked increment of chromatin cleavage and LDH release (Table 2). In contrast to renal epithelial cells, 24-h exposure to ouabain did not change the morphology and survival of VSMCs from rat aorta. Neither C11-MDCK cells nor VSMCs were affected by 24-h inhibition of Na^+,K^+-ATPase in K^+-free medium. In K^+-free medium, ouabain killed MDCK cells but did not manifest any cytotoxicity on VSMCs (Fig. 2, Table 2).

Actions of Ouabain and K^+-Free Medium on Cell Volume

Keeping in mind that 2-h incubation with ouabain caused full-scale loss of Na_i^+ and gain of K_i^+ (Fig. 1), we measured cell volume modulation in 3 h of Na^+,K^+-ATPase

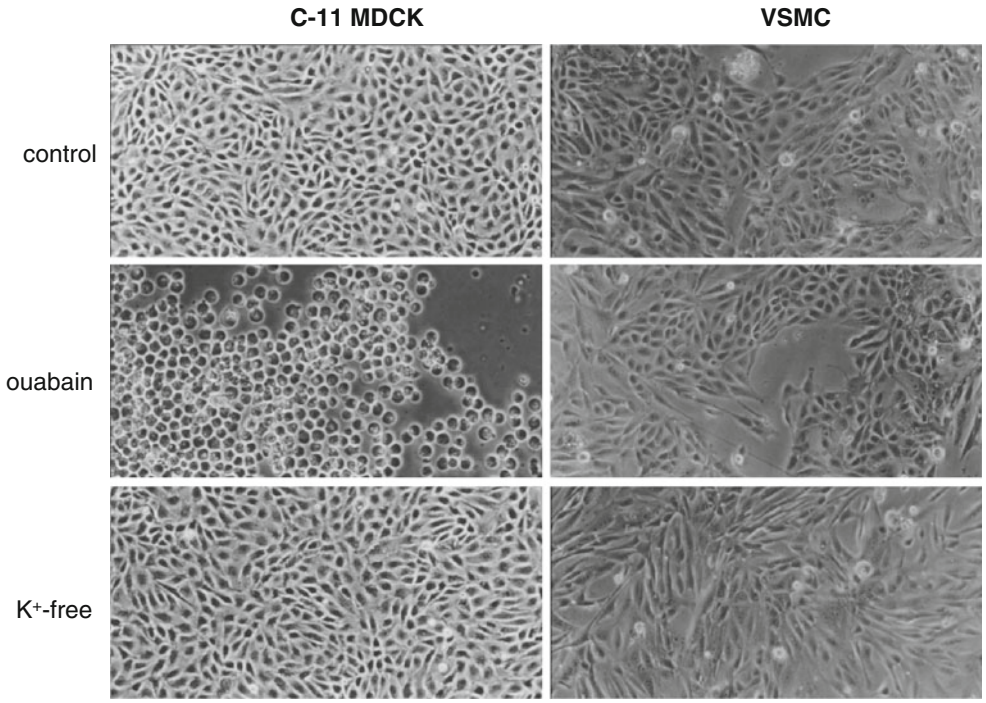

Fig. 2 Phase-contrast microscopy of renal epithelial cells (C11-MDCK) and VSMCs treated for 24 h with ouabain and K^+-free medium. Ouabain was added at concentrations of 3 and 3,000 μM in C11-MDCK cells and VSMCs, respectively

Table 2 Effect of ouabain and K^+-free medium on attachment, chromatin cleavage and LDH release from renal epithelial cells (C11-MDCK) and VSMCs

Medium	C11-MDCK			VSMC		
	Detached cells (%)	Chromatin cleavage (%)	LDH release (%)	Detached cells (%)	Chromatin cleavage (%)	LDH release (%)
Control (medium A)	6 ± 3	5 ± 2	7 ± 3	4 ± 1	3 ± 1	9 ± 4
Medium A + ouabain[a]	78 ± 9	27 ± 4	89 ± 7	5 ± 3	3 ± 2	6 ± 2
K^+-free medium	5 ± 4	4 ± 4	9 ± 6	7 ± 4	6 ± 4	10 ± 5
K^+-free medium + ouabain[a]	88 ± 11	33 ± 6	93 ± 5	3 ± 2	5 ± 3	7 ± 4
$P_{1,2}$	<0.001	<0.01	<0.001	NS	NS	NS
$P_{3,4}$	<0.001	<0.02	<0.001	NS	NS	NS

Cell attachment, chromatin cleavage and LDH release were measured in 24-h incubation of cells in control or K^+-free medium with or without ouabain

NS nonsignificant

[a] In experiments with C11-MDCK cells and VSMCs, ouabain was added at concentrations of 3 and 3,000 μM, respectively. Total content of cells, total LDH content and total amount of [^{3}H]-thymidine-labeled DNA were considered as 100%. Means ± SE are reported from experiments performed in quadruplicate (cell attachment) or triplicate (chromatin cleavage and LDH release)

inhibition. Table 3 reports that suppression of Na^+,K^+-ATPase activity by ouabain or in K^+-free medium increased the volume of C11-MDCK cells by 20 and 13%, respectively. Slight swelling was also detected in ouabain-treated VSMCs. The addition of ouabain to K^+-free medium augmented C11-MDCK cell volume by ~45%; however, these differences were not statistically significant.

Previously, we observed that the death of ouabain-treated MDCK cells might be resolved by attachment assay in 5 h with maximal increments of chromatin cleavage and capase-3 activity within 10–12 h of ouabain addition (Pchejetski et al. 2003; Akimova et al. 2009). Keeping these data in mind, in additional experiments, we extended incubation time to ~7 h and measured cell volume a few minutes before cell death. We noted that 5–10 min before detachment of ouabain-treated C11-MDCK cells their volume was increased by ~30–40% (Fig. 3; Table 4). These numbers are consistent with ~30% elevation of the

Table 3 Volume of renal epithelial cells (C11-MDCK) and VSMCs at 3 h of incubation with ouabain or K^+-free medium

Medium	Cell volume (%)	
	C11-MDCK	VSMC
Control (medium A)	104 ± 5 ($n = 4$)	101 ± 3 ($n = 4$)
Medium A + ouabain[a]	120 ± 6 ($n = 7$)	113 ± 7 ($n = 5$)
K^+-free medium	113 ± 3 ($n = 4$)	ND
K^+-free medium + ouabain[a]	145 ± 25 ($n = 4$)	ND

[a] In experiments on C11-MDCK cells and VSMCs, ouabain was added at concentrations of 3 and 3,000 μM, respectively. Means ± SE obtained from n independent experiments are reported

ND values not determined

Table 4 Volume of ouabain-treated C11-MDCK cells a few minutes before detachment

	Cell volume (%)	Time of cell volume measurement (min)	Time of cell detachment (min)
Exp. 1	141	260	265
Exp. 2	134	345	348
Exp. 3	156	313	321
Exp. 4	138	422	425
Exp. 5	129	307	311
Means ± SE	140 ± 9	329 ± 54	334 ± 53

Data obtained in five independent experiments. For more details, see Fig. 3

^{14}C-urea available space documented in C7-MDCK cells at 6 h of ouabain addition (Pchejetski et al. 2003).

Cell Volume and LDH Release in Hypotonic Medium

To examine whether the modest swelling documented in ouabain-treated C11-MDCK cells (Fig. 3; Tables 3, 4) is sufficient to induce plasma membrane rupture, we compared the kinetics of volume expansion and LDH release in cells subjected to severe hypotonic shock. We discerned that two- to threefold elevation of C11-MDCK volume was achieved in ~2–3 min, whereas maximal increment (approximately fivefold) was detected at ~10 min of attenuation of medium osmolality from ~310 to 15 mOsm (Fig. 4). In parallel experiments, we measured LDH release in 3- and 10-min exposure of C11-MDCK cells to hypotonic medium. We documented negligible LDH release in 3 min that contrasted with full-scale LDH release seen in 10 min of hypotonic shock (Table 5).

Cell Volume Modulation Triggered by Plasma Membrane Permeabilization

Recently, we reported that mammalian cells with permeabilized plasma membranes behave like osmometers, indicating an important contribution of cytoplasm hydrogel in osmosensing (Fels et al. 2009). Using dynamic molecular simulation and conductivity measurements, Vrbka et al. (2006) demonstrated that Na^+ binds at least twice as strongly as K^+ to the surface of any protein under investigation. To verify the impact of distinct Na^+ and K^+ interaction with cytoplasmic proteins on the swelling of ouabain-treated cells, we studied the outcome of $[Na^+]/[K^+]$ ratio modulation on the volume of permeabilized cells with the protocol shown in Fig. 5. As predicted, partial dissipation of Gibbs-Donnan equilibrium caused by substitution of extracellular Na^+ with K^+ resulted in C11-MDCK and VSMC swelling by 14 and 30%, respectively (Table 6). In both cell types, swelling was further increased by plasma membrane permeabilization with

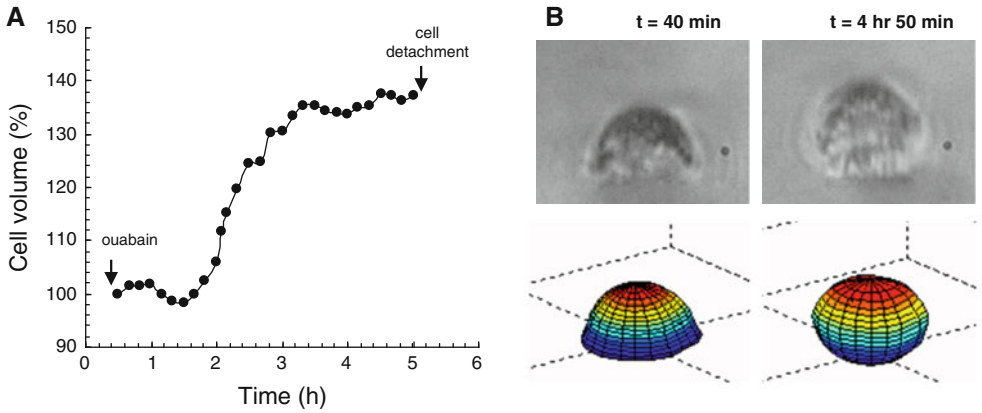

Fig. 3 **a** Representative kinetics of volume modulation by 3 μM ouabain in C11-MDCK cells. The times of ouabain addition and cell detachment are shown by *arrows*. **b** *Top* Side-view images of the cell at 40 min and 4 h, 50 min of ouabain addition. *Bottom* Perspective view of 3D models corresponding to the same cell at 40 min and 4 h, 50 min of ouabain addition, which were produced by the DISUR technique

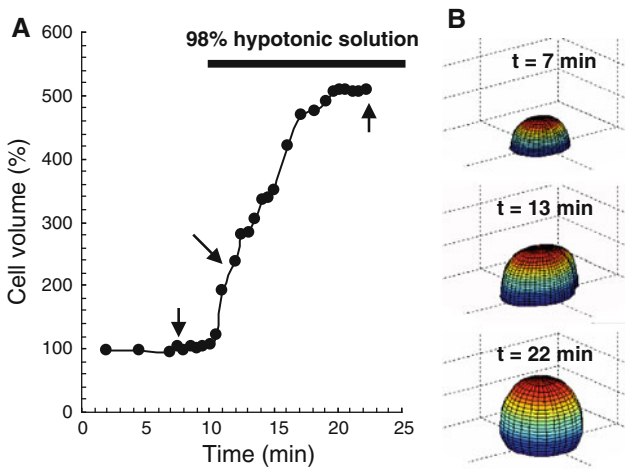

Fig. 4 **a** Representative kinetics of C11-MDCK cell volume modulation by hypotonic medium containing 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 10 mM HEPES-NaOH (pH 7.4). **b** Perspective view of 3D models corresponding to time points shown by *arrows* (~7, 13 and 22 min), which were produced by the DISUR technique

Table 5 Cell volume and LDH release in C11-MDCK cells treated with hyposmotic medium

	Cell volume (%)	LDH release (%)
10-min treatment in isosmotic medium A	103 ± 7 (n = 3)	12 ± 6 (n = 4)
3-min treatment in hyposmotic medium	248 ± 62 (n = 5)	17 ± 8 (n = 4)
10-min treatment in hyposmotic medium	494 ± 26 (n = 5)	91 ± 5 (n = 4)
$P_{1,2}$	NS	NS
$P_{1,3}$	<0.0001	<0.0005

Hypotonic medium contained 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 10 mM HEPES-NaOH (pH 7.4). Initial cell volume and total LDH content were considered as 100%. Means ± SE obtained from n independent experiments are reported

NS nonsignificant

digitonin. These data are consistent with our previous results in digitonin-treated A549, 16HBE14o and Hl-60 cells (Fels et al. 2009). We did not observe any significant impact of K^+ substitution with Na^+ on the volume of permeabilized VSMCs and C11-MDCK cells (Table 6).

Discussion

In accordance with the thermodynamic model, ouabain and other CTSs trigger the dissipation of Gibbs-Donnan equilibrium and electrical membrane potential, which, in turn, may result in necrotic volume increase and cytotoxic plasma membrane rupture (Macknight and Leaf 1977; Armstrong 2003). Supporting data were mainly obtained in hepatocytes subjected to ATP depletion via hypoxia, oxidative stress or inhibition of oxidative phosphorylation. Employing electronic cell sizing or light scattering with FACScan, Carini et al. (1999) found that suspended rat hepatocytes subjected to ATP depletion swelled in two phases: initial ~15% swelling with preserved plasma membrane integrity and terminal 30–40% swelling, followed by loss of the plasma membrane barrier to propidium iodide and the accumulation of dead cells. They also noted that the swelling and death of hepatocytes were abolished in Na^+-depleted medium (Carini et al. 1995).

It should be underlined, however, that the actions of CTS on cell volume are cell type-specific. Thus, 10–30 min of exposure to ouabain led to swelling of guinea pig ventricular myocytes (Takeuchi et al. 2006) and leech Retzius neurons (Dierkes et al. 2006) but had no effect on the volume of rabbit ventricular myocytes (Drewnowska and Baumgarten 1991), primary cultured rat hepatocytes (Russo et al. 1994), rat astrocytes (Kimelberg and Ransom 1986), Jurkat cells (Nobel et al. 2000) and A6 cells (Granitzer et al. 1994) while evoking shrinkage of cultured embryonic chick cardiac myocytes (Smith et al. 1993) and *Helix aspersa* neurons (Alvarez-Leefmans et al. 1992). In perfused rabbit cortical collecting tubules, ouabain triggered transient swelling that was followed by a regulatory volume decrease, with a final volume 15% below control values (Strange 1990).

Side by side with the cell type-specific impact of intracellular monovalent ions and organic osmolytes on cell volume adjustment (Lang et al. 1998; Mongin and Orlov 2001; Hoffmann et al. 2009), the controversial results obtained in these studies can be at least partially explained by the absence of reliable methods for long-lasting monitoring of cell volume dynamics in adherent cells. Indeed, light scattering can be applied for relative cell volume estimation in suspended cells with homogenous shape. Electronic cell sizing by a Coulter counter can be undertaken for volume measurement of suspended cells. Importantly, harsh trypsinization routinely used for cell volume estimation in substrate-attached cells might be sufficient per se to perturb cell volume (Andersen et al. 2005). Moreover, detachment of epithelial and endothelial cells causes so-called anoikis, i.e., the mode of cell death accompanied by shrinkage (Akimova et al. 2008). Quantification of cell volume from planar area measurements cannot be applied to attached cells, whose volume perturbations are mainly caused by alterations of cell height rather than planar area (Boudreault and Grygorczyk 2004; Groulx et al. 2006; Fels et al. 2009). Alternative techniques utilizing membrane-permeable radiotracer compounds, such as [^{14}C]-urea and or methyl-D-[^{14}C]glucose, require prolonged preincubation, have limited accuracy and cannot be employed to investigate rapid volume disturbances. Some of these constraints can be overcome by cell volume measurements based on isosbestic

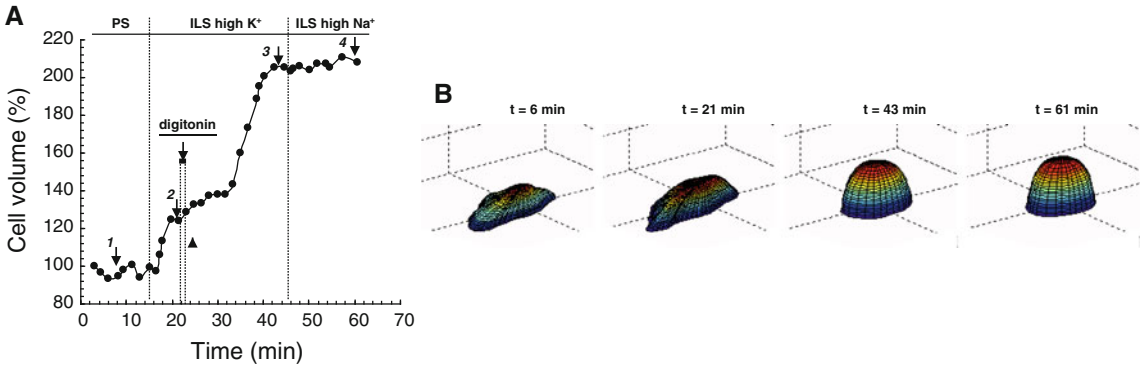

Fig. 5 **a** Representative kinetics of C11-MDCK cell volume modulation after permeabilization with digitonin and inversion of the $[Na^+]/[K^+]$ ratio. Intracellular-like solution (ILS) contained 1,200 mM KCl, 5 mM $MgCl_2$, 1 mM ATP, 11 mM dithiotreitol, 25 mM imidazole and 10 mM HEPES-KOH (pH 7.1). Digitonin, 5 μg/ml, was added for 5 min. In high-Na ILS, KCl was substituted with an equimolar amount of NaCl. *Arrow numbers* (*1–4*) correspond to moments of cell volume measurement shown in Table 6. **b** Perspective view of 3D models corresponding to time points shown by *arrows* (~6, 21, 43 and 61 min), which were produced by the DISUR technique

Table 6 Effect of permeabilization with digitonin and inversion of the $[Na^+]/[K^+]$ ratio on the volume of renal epithelial cells (C11-MDCK) and VSMCs

Medium	Cell volume (%)	
	C11-MDCK ($n = 6$)	VSMC ($n = 4$)
1. Control (medium A)	97 ± 6	103 ± 9
2. Intracellular-like solution (ILS)	114 ± 16	130 ± 19
3. ILS + digitonin	156 ± 31	213 ± 32
4. High-Na ILS	157 ± 31	261 ± 47
$P_{1,3}$	NS	<0.02
$P_{3,4}$	NS	NS

ILS contained 120 mM KCl, 5 mM $MgCl_2$, 1 mM ATP, 11 mM dithiotreitol, 25 mM imidazole, 10 mM HEPES-KOH (pH 7.1). Digitonin, 5 μg/ml, was added for 5 min. In high-Na ILS, KCl was substituted with an equimolar amount of NaCl. Italicized numbers in the left column correspond to the numbers of arrows in Fig. 5, indicating time of cell volume measurement. Initial cell volume values were considered as 100%. Means ± SE obtained from *n* independent experiments are reported

fluorescence registration of dyes, such as fluo-2, calcein or BCECF. It should be underlined that such methods assume the homogenous distribution of optically active molecules inside cells, their negligible photobleaching as well as minimal side effects that may be evoked by the production of reactive oxygen species (Hamann et al. 2002; Solenov et al. 2004). In addition, leakage of these compounds as well as tetraethylammonium cations, measured by ion-selective microelectrodes (Reuss 1985), complicates their use for comparative analysis of the long-lasting kinetics of cell volume modulation triggered by CTS.

Here, we adopted the DISUR technique, developed in our previous investigation (Boudreault and Grygorczyk 2004), to compare the kinetics of volume modulation by ouabain in CTS-sensitive C11-MDCK cells and CTS-resistant VSMCs. Data obtained in these experiments strongly suggest that swelling-mediated plasma membrane rupture does not contribute to the cytotoxic action of ouabain. Indeed, full-scale inversion of the $[Na^+]_i/[K^+]_i$ ratio in C11-MDCK and VSMCs was apparent within 2 h of ouabain addition (Fig. 1). At this time point, the volume of ouabain-treated C11-MDCK cells and VSMCs increased by 16 and 12%, respectively (Table 3). We also noted that, like ouabain, inhibition of Na^+,K^+-ATPase in K^+-free medium resulted in the sharp inversion of the $[Na^+]_i/[K^+]_i$ ratio and modest swelling of C11-MDCK cells (Table 3). However, in contrast to ouabain, we did not observe any cytotoxic actions of K^+-free medium in C11-MDCK cells (Fig. 2, Table 2). This intriguing finding was initially documented in C7-MDCK cells by Pchejetski et al. (2003) and subsequently confirmed by Contreras et al. (2004). Studying digitonin-treated cells (Fig. 5; Table 6), we found that Na^+,K^+-ATPase inhibition led to modest cell swelling via elevation of the intracellular content of osmolytes and osmotically obliged water rather than a direct effect of the $[Na^+]_i/[K^+]_i$ ratio on protein–protein interactions and the volume of cytoplasm hydrogel. We extended incubation time with ouabain to 7 h and observed that 5–10 min before detachment of ouabain-treated C11-MDCK cells their volume was increased by ~30–40% (Fig. 3; Table 4). Importantly, LDH release from hyposmotically swollen cells was triggered when C11-MDCK cell volume was augmented by approximately fivefold but was undetectable when their swelling was limited to two- to threefold (Table 5). These data are in accordance with our previous report showing that, because of intracellular vesicle insertions, A549, 16HBE14o-, NIH 3T3 and Chinese hamster ovary cells have extremely large plasma membrane reserves, which allows them to increase plasma

membrane surface area and volume approximately four- and 10-fold, respectively, in the absence of lytic rupture (Groulx et al. 2006).

The mechanisms underlying Na_i^+,K_i^+- and cell volume-independent plasma membrane disintegration in CTS-treated renal epithelial and endothelial cells remain poorly understood. During the last two decades, dozens of research teams have reported Na_i^+,K_i^+-independent effects of CTS, including caveolin-mediated interaction of α-Na^+,K^+-ATPase with the membrane-associated nonreceptor tyrosine kinase Src, which, in turn, activated diverse signaling pathways, such as extracellular responsive mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), PI3K-dependent protein kinase B, phospholipase C (PLC), $[Ca^{2+}]_i$ oscillations and increased production of reactive oxygen species (ROS) (for reviews, see Xie 2003; Aperia 2007; Schoner and Scheiner-Bobis 2007). In a previous investigation, we failed to detect any involvement of PLC, Ca_i^{2+} and ROS in the death of ouabain-treated MDCK cells (Akimova et al. 2005b). We noted, however, that the death of CTS-treated MDCK cells was suppressed by very modest intracellular acidification (Akimova et al. 2006b), p38 MAPK inhibitors (Akimova et al. 2009) and transfection with CTS-resistant α1-Na^+,K^+-ATPase (Akimova et al. 2010). The relative impact of ubiquitous and tissue-specific Na^+,K^+-ATPase subunits in triggering of the death signal by CTS as well as de novo expressed H_i^+-sensitive genes in progression of the cell death machinery should be examined further.

In conclusion, cell volume measurements in single substrate–attached cells with the recently developed DISUR technique show that the rupture of plasma membranes in ouabain-treated C11-MDCK cells was not directly caused by cell swelling triggered by Na^+,K^+-ATPase inhibition and inversion of the $[Na^+]_i/[K^+]_i$ ratio.

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